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CRISPR/Cas9 facilitates rapid generation of constitutive forms of transcription factors in *Aspergillus niger* through specific on-site genomic mutations resulting in increased saccharification of plant biomass

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Abstract

The CRISPR/Cas9 system has been successfully applied for gene editing in filamentous fungi. Previous studies reported that single stranded oligonucleotides can be used as repair templates to induce point mutations in some filamentous fungi belonging to genus *Aspergillus*. In *Aspergillus niger*, extensive research has been performed on regulation of plant biomass degradation, addressing transcription factors such as XlnR or GaaR, involved in (hemi-)cellulose and pectin utilization, respectively. Single nucleotide mutations leading to constitutively active forms of XlnR and GaaR have been previously reported. However, the mutations were performed by the introduction of versions obtained through site-directed or UV-mutagenesis into the genome. Here we report a more time- and cost-efficient approach to obtaining constitutively active versions by application of the CRISPR/Cas9 system to generate the desired mutation on-site in the *A. niger* genome. This was also achieved using only 60-mer single stranded oligonucleotides, shorter than the previously reported 90-mer strands. In this study, we show that CRISPR/Cas9 can also be used to efficiently change functional properties of the proteins encoded by the target gene by on-site genomic mutations in *A. niger*. The obtained strains with constitutively active XlnR and GaaR versions resulted in increased production of plant biomass degrading enzymes and improved release of D-xylose and L-arabinose from wheat bran, and D-galacturonic acid from sugar beet pulp.

Keywords: CRISPR/Cas9; filamentous fungi; constitutive transcription factor; CAZyme

1. Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) system originates from bacterial and archaeal immune systems. Cas proteins play a role in foreign sequence acquisitions, as well as disruption of exogenous DNA through endonuclease activity of some of these proteins, such as Cas9 [1]. In the CRISPR/Cas9 system, Cas9 forms a complex with a CRISPR RNA (crRNA) [2], which originates from exogenous protospacer sequences, and a trans-activating CRISPR RNA (tracrRNA) [3]. Due to crRNA-protospacer homology, the Cas9-crRNA-tracrRNA complex will be directed to the target locus, where the Cas9 endonuclease interacts with the target DNA strand through a protospacer adjacent motif (PAM), unwinds the DNA strand, and performs a double-strand break three nucleotides upstream of the PAM [4]. This system was adapted for genetic engineering using designed synthetic single-guide RNAs instead of the original crRNA-tracrRNA complex [5] and it has been successfully applied in a variety of eukaryotic organisms [6-8], including efficiently plant biomass degrading filamentous fungi [9]. However, its application has mainly focused on the inactivation of genes through deletions, point mutations or on the insertion of genes at specific loci [9-12].

Plant biomass is the most abundant carbon source on earth and it consists mainly of plant cell wall polysaccharides (cellulose, hemicelluloses and pectin), and the aromatic polymer lignin. These polymers form a complex network, ensuring the strength and rigidity of plant cells [13]. The complex structure of plant biomass requires a broad set of hydrolytic and oxidative enzymes to degrade it. Filamentous fungi are efficient plant biomass degraders due to their ability to produce and secrete large amounts of Carbohydrate Active enZymes (CAZymes, www.cazy.org [14]). Fungal enzymes also have large variety of applications in many industrial fields such as food and feed, pulp and paper or textile and detergent industries [15].

The production of enzymes required for plant biomass degradation is regulated by transcription factors, which can act as transcriptional activators or repressors [16]. Many transcription factors have been described in ascomycetous fungal model organisms such as *Neurospora crassa*, and in organisms involved in industrial applications such as *Aspergillus niger, Aspergillus oryzae* and *Trichoderma reesei* [17].

The xylanolytic transcription factor XlnR from *A. niger* was the first described fungal regulator involved in (hemi-)cellulose utilization [18]. It was also shown that a single V756F point mutation in the C-terminal region of the *xlnR* gene results in a fully active transcription factor, even under repressing conditions [19]. Hasper et al. suggested that mutations in the C-terminal region of XlnR

disturb a putative inhibitory domain, which would normally turn this transcription factor into an inactive form [19]. A similar A871V point mutation in the C-terminal region of the *Penicillium oxalicum* XlnR ortholog also resulted in enhanced expression of lignocellulolytic enzymes [20].

The pectinolytic transcription factor GaaR was also reported to show constitutive activity caused by a single point mutation in *A. niger* [21]. The endogenous *gaaR* gene was deleted and replaced with a DNA construct carrying a W361R point mutation. Alazi et al. [21] proposed that this mutation disrupts the interaction between GaaR and its repressor, GaaX [22], under non-inducing conditions.

So far, attempts to generate constitutively active transcription factor mutants involved either in site-directed mutagenesis of the target gene and its insertion in a specific genomic loci [19], the deletion of entire C-terminal regions of the target genes [23], or the insertion of a mutant allele in the deleted locus of the endogenous gene [21]. These are relatively labor-intensive approaches, which also may cause subtle additional changes at the site of integration or deletion that could further effect the phenotype. To demonstrate the versatility of CRISPR/Cas9-mediated genome editing, beyond the generation of loss-of-function deletions or point mutations and gene insertions, in this study we applied it to the generation of specific point mutations on site in the native genomic copy of *xlnR* and *gaaR*, resulting in the previously reported constitutively active versions of the regulators. The exoproteomes of the mutant strains were evaluated by SDS-PAGE and enzyme activity analyses, and their ability to saccharify crude plant biomass substrates was assessed, to confirm the functionality of the mutated versions of the regulators.

2. Material and methods

2.1 Strains, media and growth conditions

Escherichia coli DH5α was used for plasmid propagation, and was grown in Luria-Bertani (LB) medium supplemented with 50 μg mL^{-1} ampicillin (Sigma Aldrich). Fungal strains used in this study were derived from the *A. niger* CBS138852 strain. The generated mutants were deposited at the culture collection of Westerdijk Fungal Biodiversity Institute under accession numbers indicated in Table 1.

Table 1. *A. niger* strains used in this study.

CBS number	Strain description	Genotype	Point mutations	Reference
CBS 138852	N593 Δ*kusA*	*cspA1*, *pyrG*$^{-}$, *kusA::amdS*		[24]
CBS 145907	XlnR V756F	*cspA1*, *pyrG*$^{-}$, *kusA::amdS*	G2330T	This study
CBS 145908	GaaR W361R	*cspA1*, *pyrG*$^{-}$, *kusA::amdS*	T1285C, C1293T	This study

Strains were grown at 30˚C in Aspergillus Minimal Medium (MM) or Complete Medium (CM) [25] supplemented with 1% D-glucose and 1.22 gL^{-1} uridine (Sigma Aldrich).

For liquid cultures, freshly harvested spores were pre-grown in CM containing 2% D-fructose and 1.22 gL^{-1} uridine for 16 h at 30˚C in a rotary shaker at 250 rpm. The mycelium was harvested by filtration through sterile cheesecloth, rinsed with MM, and approximately 2.5 g (wet weight) mycelium was transferred into 50 mL MM containing 0.45% D-fructose (corresponding to 25 mM) or 2% D-fructose, 1% wheat bran (WB) or 1% sugar beet pulp (SBP). Supernatant samples were taken after 24 h incubation at 30˚C in a rotary shaker at 250 rpm. The samples were centrifuged (20 min, 3220 × *g,* 4˚C) and cell-free supernatant samples were stored at -20˚C until further processing.

2.2 Construction of mutant strains

The ANEp8-Cas9-*pyrG* plasmid, which contains the autonomous fungal replicating element AMA1,*pyrG* as selection marker, *cas9* gene and the guide RNA (gRNA) expression construct under the control of the proline transfer ribonucleic acid ($tRNA^{Pro1}$) promoter, was used in this study [26]. The ANEp8-Cas9-*pyrG* plasmids (Fig. S1) were constructed according to the protocol described by Song et al. [26]. The gRNA sequences were predicted using Geneious 11.1.4 software (https://www.geneious.com), and P1-P4 primers (Table S1) were used for the amplification of the gRNA expression constructs, which were cloned into the ANEp8-Cas9-*pyrG*

plasmids and subsequently transformed into *E. coli*. Correct clones were identified by PCR amplification of the gRNA coding region by using the Fw-screen and Rev-screen primers (Table S1). All primers used in this study were ordered from Integrated DNA Technologies, Inc. (IDT, Leuven, Belgium).

Single-stranded DNA 60-mer, 90-mer or 200-mer oligonucleotides carrying specific point mutations (Table S2) (IDT, Leuven, Belgium) were designed to be used as repair templates to repair the double stranded DNA breaks caused by Cas9. Multiple templates were used for the introduction of GaaR W361R mutation, including templates with extended length or multiple point mutations flanking the target site to facilitate a successful T → C transition in the nucleotide position 1285.

A. niger protoplasting and transformation were performed as described by Kowalczyk et al. [27], with minor modifications. One µg ANEp8-Cas9-*pyrG* plasmid, together with 5 µg of each corresponding repair template were used for each transformation. Putative mutant strains were purified by two consecutive single colony streaking, followed by cultivation on uridine-containing plates in order to remove the self-replicating AMA1 plasmid [28]. Candidates carrying the expected mutations were subsequently grown on medium containing 5-fluoro-orotic acid (5-FOA) in order to screen for colonies, which have lost the ANEp8-Cas9-*pyrG* plasmid. All *A. niger* mutants were confirmed by Sanger sequencing (Macrogen Europe, Amsterdam, the Netherlands) (Fig. S2) using the sequencing primers listed in Table S1.

2.3 SDS-PAGE and enzyme activity assays

Culture filtrates of the control and mutant strains grown in media containing WB or SBP for 24 h were used to evaluate the produced extracellular CAZymes and their activities.

Twelve µL of the culture filtrates was added to 4 µL loading buffer (10% of 1 M Tris–HCl, pH 6.8; 42% Glycerol, 4% (w/v) SDS; 0.02% (w/v) bromophenol blue; 4% of 14.7 M mercaptoethanol), incubated at 85˚C for 15 min, ice-cooled for 2 min and centrifuged at ~ 10,000 × *g* for 2 min. Finally, 10 µL were loaded onto 12% (w/v) acrylamide SDS-PAGE gels calibrated with PageRuler Plus prestained protein marker (Thermo Scientific), and silver stained [29] and documented using HP Scanjet G2410 scanner. All samples were evaluated in biological duplicates.

Enzyme activities were evaluated based on colorimetric para-nitrophenol (pNP) assays. Ten µL supernatant samples were mixed with 10 µL 0.1% 4-nitrophenyl β-D-xylopyranoside (for β-

xylosidase activity), 0.1% 4-nitrophenyl β-D-galactopyranoside (for β-1,4-D-galactosidase activity) or 0.1% 4-nitrophenyl α-L-arabinofuranoside (for α-L-arabinofuranosidase activity) substrates, 50 µL 50 mM NaAc (pH 5) and 30 µL demineralized water in a final volume of 100 µL. β-xylosidase and β-1,4-D-galactosidase activities were measured after 1 h incubation at 30˚C, while the α-L-arabinofuranosidase activity was measured after 30 min incubation at 30˚C. The reactions were stopped by the addition of 100 µL of 0.25 M Na_2CO_3 and absorption values were measured at 405 nm wavelength using FLUOstar OPTIMA (BMG Labtech). All measurements were performed by using both technical and biological triplicates. Differences in enzyme activities were determined using Student's two-tailed type II *t*-test. Significance was regarded as $p < 0.05$.

2.4 Saccharification tests

Saccharification reactions were performed in 96-well flat bottom microtiter plates. Each reaction contained 20 µL culture filtrate mixed with 50 mM sodium citrate (pH 5) containing 3% WB or 3% SBP in a final volume of 250 µL. The reactions were incubated for 6 h at 30˚C and 400 rpm. Reactions were stopped by heat inactivation of enzymes for 15 min at 95˚C. Plates were centrifuged for 20 min at 3220 × *g*, and the supernatants were 10-fold diluted in MilliQ water prior to analysis. The experiment was performed using biological and technical triplicates.

Monosaccharides were analyzed from peak areas in HPAEC-PAD (Dionex ICS-5000 + system; Thermo Scientific) equipped with CarboPac PA1 column (2×250 mm with 2×50 mm guard column; Thermo Scientific). The column was pre-equilibrated with 18 mM NaOH followed by a multi-step gradient: 0-20 min: 18 mM NaOH, 20-30 min: 0-40 mM NaOH and 0-400 mM sodium acetate, 30-35 min: 40-100 mM NaOH and 400 mM to 1 M sodium acetate, 35-40 min: 100 mM NaOH and 1 M to 0 M sodium acetate followed by re-equilibration of 18 mM NaOH for 10 min (20°C; flow rate: 0.30 mL/min). Between 5-250 mM D-glucose, D-xylose, L-arabinose and D-galacturonic acid (Sigma-Aldrich) were used as standards for quantification. Blank samples containing 3% WB or SBP, without the addition of culture filtrates were measured and the values were subtracted from each corresponding saccharification test result in order to exclude the amount of free sugar already present in the experimental condition. Differences in saccharification efficiency were determined using Student's two-tailed type II *t*-test. Significance was regarded as $p < 0.05$.

3. Results and discussion

3.1 CRISPR/Cas9 facilitates efficient on-site functional mutations

In order to achieve precise point mutations without unspecific genomic alterations, such as random insertions or deletions, we used *A. niger* Δ*kusA* as receptor strain for all our transformations [24]. Due to the lack of non-homologous end joining (NHEJ) DNA repair pathway caused by the *kusA* deletion, Cas9 double strand breaks must be repaired with a repair template homologous to the target DNA region, facilitating the implementation of short templates carrying specific point mutations. The repaired DNA strand may still serve as a target region for further Cas9 cutting events, so due to the lack of NHEJ, it is important to introduce intended alterations of the protospacer or PAM sequence in order to avoid further double strand DNA breaks, leading to the death of the mutant colonies.

Nødvig et al. previously described that 90-mer single stranded oligonucleotides could be used for successful introduction of nonsense codons into the pigmentation gene *yA*, *alba* and *wA* of *A. nidulans, A. oryzae* and *A. niger*, respectively. It was also shown that the DNA repair did not show any preference for the targeted sense or anti-sense strand [30]. Based on this, we decided to use repair templates complementary to the anti-sense strand of the target DNA.

First, we performed a single GTC → TTC nucleotide mutation in the *xlnR* coding region, resulting in a valine-756-phenylalanine (V756F) mutation [19]. The gRNA sequence (P3-XlnR, P4-XlnR, Table S1) closest to the nucleotide of interest was predicted by Geneious. The 90-mer oligonucleotide repair template (XlnR repair template, Table S2) did not require any additional point mutations, since the target codon was also part of the PAM sequence, ensuring that the Cas9 endonuclease would not be able to re-bind and cut the repaired sequence anymore. After fungal transformation, three randomly chosen candidates were sequenced in their CRISPR/Cas9 target site (Fig. 1A). All three candidates were shown to be correct and candidate 3 was randomly selected for further phenotype evaluation.

To obtain a constitutively active GaaR [21], a T → C transition in a TGG codon is required, resulting in a tryptophan-361-arginine (W361R) mutation. The gRNA sequence (P3-GaaR, P4-GaaR, Table S1) with the highest on-target activity was predicted by Geneious based on the experimentally determined predictive model proposed by Doench, et al. [31]. Contrary to the *xlnR* point mutation design, an additional mutation was required in order to avoid re-cutting of the repaired target strand by the CRISPR/Cas9 system. Previous studies reported that the

CRISPR/Cas9 system shows tolerance to mismatches in the protospacer sequence [32], which led us to alter the PAM sequence with a G → C silent mutation.

A 90-mer single stranded oligonucleotide was designed to introduce the intended point mutations, interspaced by 52 nucleotides (GaaR repair template 1, Table S2). After transformation, four randomly selected candidates were submitted for sequencing. Sequencing results (Fig. 1B) showed the intended alteration of the PAM sequence, although the W361R mutation did not take place. The same transformation was attempted with a longer 200-mer oligonucleotide repair template (GaaR repair template 2, Table S2). All sequenced colonies carried only the PAM sequence altering mutation (data not shown). Both attempts suggest that the repair templates were not entirely incorporated, excluding the W361R mutation, most likely due to the large distance between the two mutated nucleotides.

Taking into account these results, a re-designed gRNA encoding sequence (P3.2-GaaR, P4.2-GaaR, Table S1) closer to the target nucleotide was performed. Similarly to the previous approach, a new repair template was designed carrying two point mutations (2 PM), but this time the mutations were interspaced by only seven nucleotides. Since the new repair template carried both the intended W361R and the PAM sequence mutations closer to each other, we decided to reduce the length of the repair template to 60 oligonucleotides, which would theoretically induce homologous recombination (GaaR repair template 2 PM, Table S2). In addition, another repair template was designed carrying five additional silent mutations (GaaR repair template 7 PM, Table S2) in order to hinder the homology of the sequence around the nucleotide of interest, to avoid the previously observed results where only the PAM sequence alteration occurred. Five transformant colonies were sequenced for each transformation, resulting in four correct mutants each (Fig. 1C). Interestingly, the 2 PM repair template resulted in one colony carrying only the PAM sequence altering mutation, where most likely a recombination happened with ≤ 12 nucleotides serving as 5'-end flanking region, suggesting that even shorter repair templates could successfully restore the damaged DNA. This would be especially relevant when CRISPR/Cas9 genome editing is performed in an *A. niger* strain of a different lineage, whose genomic DNA sequence is likely not fully identical. The colony 2 PM 1 was selected for further phenotypic evaluation.

3.2 Constitutive versions of XlnR and GaaR result in elevated enzyme levels

Two crude plant biomass substrates were chosen for phenotypic characterization of XlnR V756F and GaaR W361R mutants. WB is rich in glucuronoarabinoxylan, suitable for characterization of a constitutive XlnR phenotype, whereas SBP has a high pectin content and was previously used for the characterization of *gaaR* deletion mutants [27, 33].

SDS-PAGE and enzyme activity assays of 24 h culture filtrates after growth of the mutants and control strain on 1% WB and 1% SBP were assessed for phenotypic characterization. SDS-PAGE results of the XlnR V756F mutant grown in 2% D-fructose, a carbon source showing low carbon catabolite repression (CCR) mediated gene repression [34], showed the presence of mainly putative endoxylanases (13-33 kDa) and β-xylosidases (122 kDa) [13] (Fig. 2A), which were not present in the control strain, demonstrating the inducer-independent constitutive action of XlnR. The xylanolytic enzymes were more abundant when the mutant was grown in medium containing 0.45% D-fructose (Fig. 2B), most likely due to the reduced CCR effect mediated by CreA compared to the 2% D-fructose culture [35, 36]. Cultivation of the XlnR V756F mutant in 1% WB medium also resulted in an increase of the major putative xylanolytic enzymes compared to the control strain (Fig. 2C), suggesting improved saccharification abilities in this mutant.

The cultivation of the GaaR W361R mutant in liquid medium containing 2% D-fructose as a sole carbon source did not result in an increased production of pectinolytic enzymes as the SDS-PAGE pattern was identical to that of the control strain (data not shown). However, the samples from 1% SBP cultures showed elevated levels of CAZymes, especially in the 35-66 kDa range (Fig. 2D), where most *A. niger* endo- and exopolygalacturonases, and pectin lyases are found [13]. The genes encoding these enzymes have been shown to be controlled by GaaR [37].

The SDS-PAGE profiles were confirmed by enzyme activity assays. β-xylosidase activity (BXL) was 53% increased in the XlnR V756F supernatant from 1% WB cultures compared to its control strain (Fig. 3A). Moreover, BXL activity in the culture filtrate of this mutant showed very similar values when cultivated in 1% SBP, while the control strain did not show any BXL activity under this condition, due to the lack of activation of XlnR (Fig. 3B). This result also proves that the XlnR V756F mutant can express its target genes in non-inducing conditions. Interestingly, the supernatant from GaaR W361R mutant showed a 31% increase in β-1,4-D-galactosidase activity (LAC) in WB medium. However, LAC activity was 63% reduced in samples of the SBP cultures, compared to the control. Since the regulation of β-galactosidase genes has been shown to be controlled by a broad range of transcription factors, including GaaR [37], the increase of LAC activity in WB medium and decrease in SBP medium may not be related to a direct constitutive GaaR effect, but rather to an altered interaction between the transcription factors controlling the

production of this activity. Finally, the XlnR V756F mutant showed 15% and 10% reduced α-L-arabinofuranosidase activity (ABF) in the WB and SBP samples, respectively. In contrast, the GaaR W361R mutant showed a 22% and 6% increase in ABF activity in WB and SBP cultures, respectively, suggesting that the constitutive GaaR rather has an (minor) activating role in the expression of *abf* genes. Overall, the low fold change values suggest that neither XlnR V756F, nor GaaR W361R play an essential role in the activation of these genes, most likely because they are mainly controlled by the arabinanolytic regulator AraR [37].

3.3 The enzyme mixtures from the constitutive regulator strains resulted in improved saccharification of wheat bran and sugar beet pulp

Saccharification tests were performed using the 24 h culture filtrates of XlnR V756F, GaaR W361R and the control strain cultured on 1% WB or 1% SBP (Fig. 4) (subsequently referred to as WB culture filtrate and SBP culture filtrate, respectively). Both crude substrates were used in order to test the phenotype of each mutant strain under inducing and non-inducing conditions.

The release of D-xylose, L-arabinose, D-galacturonic acid and D-glucose from 3% WB and 3% SBP by the WB and SBP culture filtrates was measured. D-xylose release from xylan is regulated by XlnR, while the release of D-xylose from pectin has been suggested to be co-regulated by XlnR and GaaR [27, 37]. The release of L-arabinose is mainly controlled by the arabinanolytic transcription factor, AraR [38], but XlnR and GaaR have also been reported to co-regulate some arabinanolytic genes [27, 37]. The release of D-galacturonic acid from pectin is regulated by GaaR [33]. Finally, D-glucose can either be released from cellulose or starch. SBP is rich in cellulose, while WB contains both cellulose and starch [39]. Since neither XlnR nor GaaR was shown to play a role in starch utilization, D-glucose release would be most likely related only to cellulose utilization, in which XlnR is involved, as first suggested by van Peij et al. [40].

Saccharification of WB (Fig. 4) showed increased release of D-xylose (Fig. 4A) and L-arabinose (Fig. 4B) by the XlnR V756F mutant for both WB and SBP culture filtrates. When the SBP culture filtrates were used, D-xylose release by XlnR V756F was especially significant compared to the control strain, which did not release D-xylose due to the lack of XlnR induction (Fig. 4A). The GaaR W361R mutant showed a similar amount of released D-xylose (Fig. 4A) compared to the control for both culture filtrates, while L-arabinose release (Fig. 4B) was similar for the WB culture filtrate, but reduced for the SBP culture filtrate. This could be associated with a competing effect

297 between GaaR and XlnR [27], also supported by the fact that the constitutive XlnR resulted in a
298 significant increase of L-arabinose release in this condition.

299 D-galacturonic acid is present in very low amounts in WB, most likely found only in thin layers of
300 pectin located under the outer and the epidermal cuticles [39, 41]. Our results show that SBP
301 culture filtrate from XlnR V756F generated the highest D-galacturonic acid release (Fig. 4C),
302 probably due to the more abundant presence of β-xylosidases catalyzing the removal of D-xylose,
303 which can decorate pectin. This could facilitate the degradation of the galacturonan backbone by
304 the pectinolytic enzymes also present in the SBP culture filtrates. Regarding D-glucose release
305 (Fig. 4D), the XlnR V756F mutant showed a similar value compared to the control strain when
306 WB culture filtrate was used. However, for the SBP culture filtrate, the XlnR V756F mutant showed
307 significantly higher D-glucose release, which is most likely related to the improved xylanolytic
308 activities, making cellulose more accessible for degradation in WB. Both GaaR W361R culture
309 filtrates showed reduced D-glucose release from WB, possibly due to an antagonistic effect
310 between GaaR and XlnR [27], resulting in decreased xylanolytic activity on hemicellulose in the
311 constitutive GaaR mutant, thus reducing cellulose degradation.

312 In case of SBP saccharification (Fig. 5), all strains released similar levels of D-xylose when WB
313 culture filtrates were used (Fig. 5A). In contrast, the XlnR V756F SBP culture filtrate showed a
314 significant improvement in D-xylose release compared to the control. Regarding L-arabinose
315 release (Fig. 5B), SBP culture filtrates showed higher saccharification efficiency compared to that
316 of WB culture filtrates. However, the mutants did not show improved L-arabinose release
317 compared to the control strain, which indicates that other transcription factors, such as AraR, have
318 a more predominant role in releasing L-arabinose from pectin. These results also suggest that
319 the XlnR V756F and/or GaaR W361R mutants has a negative effect over AraR-mediated L-
320 arabinose release from this substrate. In contrast, D-galacturonic acid release was significantly
321 improved by both GaaR W361R culture filtrates (Fig. 5C), especially in the case of WB culture
322 filtrates, where the control strain released only a minimal amount of D-galacturonic acid due to
323 the lack of GaaR induction. Finally, D-glucose release was similar for the WB culture filtrate
324 samples, while both mutants showed a slight decrease compared to the control when SBP culture
325 filtrates were used (Fig. 5D).This suggests that neither XlnR nor GaaR have a major influence on
326 cellulose degradation under these conditions.

327 Overall, the release of D-xylose and L-arabinose from WB was improved by the XlnR V756F
328 mutant. D-xylose release from both WB and SBP using the non-inducing condition for XlnR (SBP
329 culture filtrate) highlights the constitutive activity of the XlnR V756F mutant. D-galacturonic acid

release from SBP was improved by the GaaR W361R mutant, highlighting the constitutive activity in case of the non-inducing WB culture filtrates. The results of D-glucose release from SBP suggests that the cellulolytic activities of each sample are comparable, which justifies the results observed in case of WB saccharification, in which xylan degradation is the bottleneck of cellulose utilization.

4. Conclusions

In this work, we demonstrate how CRISPR/Cas9 genome editing can be used to efficiently modify the functionality of transcriptional regulators in *A. niger* by generating on-site mutation in the native copy of the corresponding genes in the genome. This also indicates that similar strategies could be used to change enzyme properties by mutating enzyme encoding genes, as well as many other functional mutations, further expanding the versatility of this genome editing approach. We used single stranded 60-mer or 90-mer oligonucleotide-mediated gene editing to generate constitutively active XlnR and GaaR transcription factors, but our data suggests that even shorter fragments could be used as templates to repair the Cas9-induced DNA strand cuts. The XlnR V756F mutant secreted a higher amount of CAZymes involved in the release of D-xylose and L-arabinose from WB confirming the functional mutation. Moreover, D-glucose release was also improved, likely facilitated by degradation of xylan, making cellulose more accessible for degradation in non-inducing conditions. Finally, the GaaR W361R mutant showed enhanced release of D-galacturonic acid from SBP. Overall, the use of CRISPR/Cas9 to generate such overproduction strains significantly reduced time and efforts compared to traditional approaches.

5. Conflict of interests

The authors declare that they have no competing interests.

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8. Footnotes

Figure 1. Sanger sequencing results of the XlnR V756F and GaaR W361R mutant candidates. A) Sequencing results of mutant candidates transformed with XlnR repair template. All three candidates showed the expected mutation. Sequences show the whole coverage of the used repair templates. Reference sequence is highlighted in black. The location of target nucleotides are highlighted in gray, while the introduced mutations are indicated in red. The PAM sequence is shown in green. The protospacer sequence is highlighted in yellow and the Cas9 cutting site is represented by a red bar. B) Sequencing results of mutant candidates transformed with GaaR repair template 1 (Table S2). All four sequenced candidates showed the introduction of the PAM sequence altering mutation. However, the W361R mutation did not occur. C) Sequencing results of mutant candidates transformed with GaaR repair template 2 point mutations (PM) or 7 PM (Table S2). Four candidates transformed with the 2 PM or 7 PM template showed the expected mutations. The 2 PM candidate 5 showed only the introduction of the PAM sequence altering mutation, while 7 PM candidate 5 showed a target sequence identical to the reference sequence. Results of (B) and (C) are aligned in order to emphasize the new guide RNA selection for the transformations using the 2 PM or 7 PM repair templates. Color codes as in (A).

Figure 2. Enzyme production analysis of XlnR V756F and GaaR W361R mutant strains. SDS-PAGE analysis of the supernatants of *A. niger* CBS138852 (control strain), XlnR V756F and GaaR W361R mutants after 24 h incubation in different culture conditions as indicated from A-D.

Figure 3. Enzyme activity of the supernatants from XlnR V756F and GaaR W361R mutant strains and from *A. niger* CBS138852. The 24 h culture filtrates originated from 1% wheat bran (WB) (A) or 1% sugar beet pulp (SBP) (B). Different enzyme activities are indicated by gray scale color codes. The values represent the mean and standard deviation of the amount of released pNP measured at 405 nm wavelength. Experiments were carried out using biological and technical triplicates. Bxl = β-xylosidase, Lac = β-1,4-D-galactosidase, Abf = α-L-arabinofuranosidase. Statistical significance is represented by (*) ($p< 0.05$).

Figure 4. Monosaccharides released from 3% wheat bran (WB) by culture filtrates from control strain *A. niger* CBS138852, and XlnR V756F and GaaR W361R mutant strains. The amount of D-xylose (A), L-arabinose (B), D-galacturonic acid (C) and D-glucose (D) released after 6 h incubations with WB or sugar beet pulp (SBP) culture filtrates are indicated by black and gray bars, respectively. Values represent the mean and standard deviation of sugar concentration indicated in millimoles (mM). Experiments were carried out using biological and technical triplicates. Statistical significance is represented by (*) ($p< 0.05$).

Figure 5. Monosaccharides released from 3% sugar beet pulp (SBP) by culture filtrates from control strain *A. niger* CBS138852, and XlnR V756F and GaaR W361R mutant strains. The amount of D-xylose (A), L-arabinose (B), D-galacturonic acid (C) and D-glucose (D) released after 6 h incubations with wheat bran (WB) or SBP culture filtrates are indicated by black and gray bars, respectively. Values represent the mean and standard deviation of sugar concentration indicated in millimoles (mM). Experiments were carried out using biological and technical triplicates. Statistical significance is represented by (*) ($p< 0.05$).